Effect of Male Age and Hormone Induction on Sperm Quality in the Critically Endangered Mississippi Gopher Frog (Lithobates sevosus)

Ashley Michelle Watt

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Effect of Male Age and Hormone Induction on Sperm Quality in the Critically Endangered Mississippi Gopher Frog (*Lithobates sevosus*)

By

Ashley Michelle Watt

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2019

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Effect of Male Age and Hormone Induction on Sperm Quality in the Critically Endangered Mississippi Gopher Frog (*Lithobates sevosa*)

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May 15th, 2019
DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows: both of my data chapters were co-authored with my supervisor, Dr. Trevor Pitcher. In each case, my co-author provided valuable feedback, helped with the project design and statistical analysis, and provided editorial input during the writing of each manuscript; however, in both cases, the primary contributions have all been by the author. Chapter Three has been prepared as a manuscript, being submitted to the Journal of Zoo Biology for publication.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

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II. Previous Publication

This thesis includes [1] original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:
Chapter [3]  Time from injection of luteinizing hormone releasing hormone affects sperm quality in the critically endangered Mississippi gopher frog (*Lithobates sevosus*); Ashley Michelle Watt \(^1\)*, Ruth Marcec\(^2\), and Trevor Edgar Pitcher\(^1,3\)  In Review

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ABSTRACT

Zoos can play a key role in *ex-situ* conservation, focused on the management of imperiled species whose survival is dependent on conservation programs to effectively breed and reintroduce individuals back into the wild. Consequently, captive bred populations rarely become self-sustained and zoos often become limited by small, ageing populations with reproductively exhausted individuals. To overcome reproductive challenges, zoos can employ exogenous hormones to induce gamete production for artificial fertilizations. Using the critically endangered Mississippi gopher frog (*Lithobates sevosus*), our research focused on these two aspects of reproduction in captivity. First, we examined the effects of age on sperm quality through the broader theory of senescence - the reduced survival or fertility with increasing age. We found that sperm quality significantly differed between age categories. Secondly, we evaluated the spermiation response and the quality of sperm release following an injection of an exogenous luteinizing hormone-releasing hormone. We found that sperm quality differed between sampling times post-hormone injection. Collectively, this thesis aimed to test age related hypotheses in the context of senescence theory, offer valuable information about hormone induction in a species of true frog, and provide feedback to zoos to help contribute to the reintroduction effort of the Mississippi gopher frog.
DEDICATION

Alice Watt

“Keep on plugging along – do your best; always be honest – difficult to do at times, but in the end, it does win. Hard work is the best you could’ve done, so be proud.”
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The Amphibian Extinction Crisis

Earth is facing its largest mass extinction in the lifetime of the planet (Wake & Vrendenberg, 2008; Ceballos et al., 2015). Anthropogenic stressors are widespread, causing global population-level extinctions at accelerated rates (Ceballos et al., 2015; Young et al., 2016). Stressors, such as habitat loss or fragmentation, over-exploitation, and disease have been causally linked to population decline (Wake & Vrendenberg, 2008). Since first becoming evident in the 1980’s, amphibian populations are now facing accelerated declines worldwide, more so than any other taxonomic group (Collins & Storfer, 2003; Gascon, 2007). Amphibians consist of three orders: anura (frogs and toads), gymnophiona (caecilians), and urodele (salamanders and newts). Research has shown that approximately 43% of the estimated 6,000 amphibian species are currently in decline and within the last decade, the amphibian extinction rate has become 200 times greater than the preceding 350 million years (Stuart et al., 2004). In response to the dramatic loss of amphibians, a variety of conservation action plans, including in-situ strategies, “the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings” (Braverman, 2014), and ex-situ conservation strategies, “the conservation of components of biological diversity outside their natural habitats” (Braverman, 2014) have been employed. Such plans have led to the establishment of captive breeding programs (CBP’s), which at this time, act as an assurance strategy for the survival of wild populations that are no longer self-sustaining (Steffen et al., 2007; Gascon et al., 2005).
Captive Breeding Programs in Zoos

In the face of overwhelming threats, zoos have become active in *ex-situ* conservation by providing a short-term solution to house imperiled amphibians at risk of extinction in the wild (Gascon et al., 2005). CBP’s often involve practices to manage reproduction, whilst maintaining genetic diversity to avoid inbreeding or genetic bottlenecks (Watson & Holt, 2001; Lacy, 2009; Schulte-Hostedde & Mastromonaco, 2015). In addition to reproduction, these programs often implement research, education, and allocate a percentage of funds to mitigate threats still existent in the wild (Gascon et al., 2005). Within CBP’s there must be a continual effort towards the successful propagation of a species for reintroduction or there is ultimately no benefit of holding an imperiled species in captivity (Griffiths & Pavajeau, 2008). However, numerous challenges exist when breeding amphibians, especially anurans, which often struggle to reproduce naturally (Kouba & Vance, 2009).

Anuran Reproductive Dysfunction

Anurans possess several life-history traits that are advantageous for captive breeding and reintroduction (Griffiths & Pavajeau, 2008). For example, anurans are external fertilizers, they often have a high fecundity, a short generation time, and they are more cost-efficient to house. However, despite being relatively suitable for breeding, anurans often experience greater levels of reproductive dysfunction in captivity (Richter et al., 2003; Griffiths & Pavajeau, 2008). The exact cause of reproductive dysfunction is unknown, though it suspected to be attributed to a number of factors, such as environmental cues, diet, stress, or inbreeding depression (Kouba et al., 2009; Poole &
Reproductive dysfunction can occur in both sexes, which can be noticeable by a lack of breeding behaviors, such as the failure to ovulate in females, and a lack of amplexus or calling in males (Kouba et al., 2009 Kouba et al., 2012a). Reproductive dysfunction may also be attributed to an animal's age, as gamete function is suspected to decline in later years (Gasparini et al., 2010). Age-associated challenges may exist in CBP’s as zoos are often limited by space for housing or are required to reduce the captive population size (Gascon et al., 2007). As a result, zoo CBP’s may be reliant on a limited number of animals available to breed, that are of lower quality, which may be influenced by their biological age.

**Reproductive Dysfunction and Ageing**

‘Ageing’ or ‘senescence’, the decline in performance and function with age (Saino et al., 2002), is often expressed as a decline in the quality of gametes as animals age (Gasparini et al., 2010). Egg quality has been shown to decline with female age, having a profound effect on fertility (Johnson & Gemmell, 2012). While less studied, evidence supports an age-dependent decline in sperm quality, which may lower fertilization success and decrease offspring viability (Hettyey et al., 2012; Johnson & Gemmell, 2012). In evolutionary biology, senescent sperm are thought to arise from processes occurring before and after meiosis. Pre-meiotic ageing may be an outcome of mutation pressure, which results from an accumulation of *de novo* mutations in the germline cells (Radwan, 2003; Hettyey et al., 2012). Consequently, older males may be less optimal to breed as their sperm may be of lower quality (Gasparini et al., 2010). Post-meiotic ageing may also occur during sperm storage, when an increase of oxidative
stress accumulates in the sperm cell causing damage to the cell’s structure (Hettyey et al., 2012).

**Study System: The Mississippi Gopher Frog**

The critically endangered Mississippi gopher frog (MGF; *Lithobates sevosus*) belongs to the family Ranidae and is considered to be the most endangered amphibian in North America and one of the most endangered species in the world (Hammerson et al., 2004a). Historically, the MGF was found along the Gulf Coastal Plain of Louisiana, Alabama, and Mississippi (Hammerson et al., 2004a; Lannoo, 2005). Over the last half century, habitat destruction and fragmentation of the longleaf pine ecosystem has extirpated the MGF from its historic range, resulting in the disappearance of animals from Alabama since 1922 and Louisiana since 1965. By 2012, the population was estimated at 100 breeding individuals, reduced to two adjacent ponds in the DeSoto National Forest, MS, USA (USFWS, 2012a, USFWS, 2012b).

Adult MGF reside year-round in the longleaf pine forest with abundant ground cover for refuge (Lannoo, 2005; Tupy, 2012). Typically, the MGF will occupy the burrow of the gopher frog tortoise, where it will remain until environmental cues stimulate a breeding event (Lannoo, 2005). Breeding sites are temporal ponds that remain dry throughout the year, and become ephemeral, quickly forming from the heavy rainfall (Lannoo, 2005; Rorabaugh, 2005). Successful reproduction is dependent on the hydroperiod, as water levels must remain sufficient for tadpoles to survive to metamorphosis (Richter et al., 2003; Rorabaugh, 2005). Consequently, yearly
environmental fluctuations, and geographic isolation create great risk for the species to become vulnerable to extinction (Richter et al., 2003; Tupy, 2012).

Recovery efforts for the species have led the US Fish and Wildlife Service (USFWS) to establish partnerships with academic and zoological institutions that aim to achieve captive propagation of the species and reintroduction (Lannoo, 2005; USFWS, 2012b). However, the MGF has not been observed to breed naturally in captivity, posing an additional challenge for recovery. In captivity, the MGF requires a variety of assisted reproductive technologies to reproduce such as, exogenous hormones to stimulate gamete production, and artificial fertilization (Poole & Grow, 2012). At this time, the development of assisted reproduction protocols for the Mississippi gopher frog are necessary in hopes to one day recover the species.

**Hormonal Regulation of Reproduction in Anurans**

*Hypothalamic-Pituitary-Gonadal Axis*

Gamete development in frogs is driven by the endocrine system that begins at the hypothalamic-pituitary-gonadal (HPG) axis (Duellman & Trueb, 1994). The HPG axis is made up of numerous structures that play a role in hormone secretion. Anurans brains are organized into distinct regions including the forebrain, consisting of the telencephalon and diencephalon; the midbrain, and the hindbrain (Duellman & Trueb, 1994). The diencephalon is mostly made up by the hypothalamus, which releases neurohormones when triggered by environmental cues (Tsai, 2011; Norris & Lopez, 2011). The HPG axis is operated by both positive and negative feedback mechanisms at each level of the axis (Tsai et al., 2005) (Figure 1).
The hypothalamus releases the neuropeptide, gonadotropin-releasing hormone (GnRH), which stimulates the pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH), which regulate the gonads (Zerani et al., 1991; Norris & Lopez, 2011). GnRH is a decapeptide that is conserved across vertebrates and exists in two distinct forms: GnRH-I and GnRH-II (Fernald & White, 1999; Tsai, 2011). GnRH-I is concentrated in the hypothalamus and thought to be the predominant stimulator of the pituitary in the amphibian brain, while GnRH-II is thought to act as a neuroendocrine regulator (Daniels & Licht, 1980; Fernald & White, 1999; Clulow et al., 2014).

Upon stimulation, the pituitary releases the gonadotropin hormones LH and FSH, which are responsible for spermiation and ovulation (Tsai, 2011). The release of LH and FSH initiate the production of androgens (testosterone) in males and estrogens (estradiol) in females (Rastogi et al., 2011; Poole & Grow, 2012). In males, LH is responsible for stimulating steroidogenesis in the testes, which controls the production and release of testosterone. In females, LH is responsible for follicle recruitment, oocyte growth, ovulation, and vitellogenin production through the production and release of estrogens and progesterone (Browne, 2006).

Use of Exogenous Hormones to Overcome Reproductive Dysfunction

Assisted reproductive technologies encompass a variety of techniques, which include exogenous hormones. Exogenous hormones can be used to initiate ovulation in females or spermiation in males by manipulating various stages of the anuran hormone cycle (Wright & Whitaker, 2001). The two most widely used exogenous hormones are: human chorionic gonadotropin (hCG) and luteinizing hormone-releasing hormone analog
(LHRHa) (Kouba & Vance, 2009). These hormones target different levels of the hypothalamic-pituitary-gonadal (HPG) axis (see figure 1) and are often classified as ‘first generation’ and ‘second generation’ hormones (Kouba et al., 2012; Clulow et al., 2014). First generation hormones, such as hCG are naturally produced by the chorionic membrane of the placenta in mammals (Johnson & Everitt, 2007). The protein has an LH-like activity that acts directly at the level of the gonads to release testosterone for spermiation in males and progesterone for ovulation in females (Kouba et al., 2012a). Second generation hormones, such as LHRHa work at the level of the brain and indirectly stimulate the gonads by acting at the pituitary to release the animals own endogenous hormones such as, luteinizing hormone (LH) and the follicle stimulating hormone (FSH) (Kouba et al., 2012a; Tsai, 2011).

Thesis Overview

Effect of Age on Sperm Quality

A decline in sperm quality with age is a common prediction of senescence-based hypotheses and empirical studies. While widely studies across taxa, there is little known on the effect of ageing on sperm quality in amphibians. The objective of this study was to investigate variation in sperm quality metrics (i.e. motility, concentration, and morphology) in the endangered Mississippi gopher frog (Lithobates sevosus) between males of three age categories. Different aged males across the species expectant lifespan (1-9 years; 1-2 years old, 3-4 years old & 8-9 years old) were chosen in an attempt to identify an optimal breeding age relevant for captive breeding programs.
Time-Post Luteinizing Hormone-Releasing Hormone

For 50 years, exogenous hormones have been used in captive breeding programs to induce a spermiation response in a variety of frog and toad species (Kouba et al., 2012). Luteinizing hormone-releasing hormone (LHRH) acts in the brain to induce a more natural endogenous hormone response. Our study (see Chapter 3) examined how sperm quality differed over time following the administration of a luteinizing hormone-releasing hormone injection. We used 11 male gopher frogs and measured sperm motility (%), progressive motility (%), velocity (µm), and concentration (10^6 cells/ml) over three sampling times (30mins, 60mins, and 120mins) post-LHRH injection.
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http://dx.doi.org/10.1016/j.geoforum.2013.09.018.


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Figure 1. The reproductive hormone cycle in amphibians. Environmental cues initiate the production of gonadotropin-releasing hormone (GnRH) in the brain. GnRH stimulates the pituitary to produce gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Gonadotropins stimulate the testes to produce testosterone, promoting the production of spermatozoa and stimulate the follicles to produce estrogen and progesterone, promoting the maturation of oocytes and ovulation (Browne, 2006).
CHAPTER 2
EFFECT OF MALE AGE ON SPERM QUALITY IN CAPTIVE-REARED ENDANGERED MISSISSIPPI GOPHER FROG (LITHOBATES SEVOSUS)

Introduction
For a growing number of species, zoos have established captive breeding programs as part of their commitment to conserve and reintroduce imperiled species back into the wild. Though not a long-term solution, in many circumstances, captivity may be the only opportunity to protect animals from direct threats such as habitat loss and disease (Gascon et al., 2007). By removing some individuals from the wild, captive breeding programs can act as an ‘insurance’ against extinction until more secured habitat is available (Gascon et al., 2007; Griffiths & Pavajeau, 2008). The central objective of captive breeding programs is to facilitate the successful propagation of imperiled species, though this often remains difficult to achieve. Generally, species struggle to reproduce in captivity, and numerous factors are suspected to contribute to this dilemma (Schulte-Hostedde & Mastromonaco, 2015). For instance, the reproductive patterns for reptiles and amphibians often correlate with environmental cues (i.e. temperature and photoperiod) (Laszlo, 1979; Norris and Lopez, 2011; Kouba et al., 2012a). In captivity, there often remains an inability to effectively replicate such cues, decreasing the likelihood of a natural reproductive event from occurring. Captive populations may also experience low reproductive success as programs must manage financial constraints and housing limitations when breeding endangered species (Gascon et al., 2007). In general, older animals may be more prevalent in breeding programs due to the complexity of acquiring new animals from a wild source population (Snyder et al., 1995). As such,
programs can be limited by the quality of animals available to breed, which may be
determined by their biological age.

‘Ageing’ or ‘senescence’, “the decline in performance and function with age”
(Saino et al., 2002), is often expressed as a decline in the quality of gametes as animals
age (Gasparini et al., 2010). For example, a literature review by Johnson & Gemmell,
(2012) reported the quality of sperm often declines with age, however this has been
shown to vary widely among taxa. By contrast, many studies have focused on female
fertility, suggesting an age-dependent decline in egg quality is the limiting factor in the
production of viable offspring (Johnson & Gemmell, 2012). Evolutionary theories of
senescence often focus on age-dependent patterns of reproduction, and an organism’s
trade-off between allocating energy amongst essential processes (i.e. growth,
reproduction, and survival) (Partridge & Barton, 1993; Radwan, 2003; Møller et al.,
2009). This concept, termed the ‘disposable soma theory’ assumes a higher investment
into reproduction earlier can compromise somatic repair later in life (Møller et al., 2009).
While less studied, evidence supports a decline in sperm quality with male age as an
outcome of life history optimization associated with mutation pressure (Radwan, 2003).
An accumulation of de novo mutations in the germline cells occurs when cells continue to
divide after sexual maturity, which may lead to a higher mutation load (Radwan, 2003;
Hettyey et al., 2012). For example, Syntin & Robaire (2001) found that male age has a
significant effect on motility, which steadily declines with male age in the Brown
Norway Rat (Rattus norvegicus).

Growing evidence suggests that an age-dependent decline in sperm quality may
have negative downstream effects on fertilization success and offspring fitness (Kidd et
al., 2001; Radwan, 2003; Johnson & Gemmell, 2012). For instance, in male fowl (*Gallus gallus domesticus*), fertilization success decreases with age as older males often ejaculate sperm with lower swimming ability (Dean et al., 2010). However, few studies have specifically investigated how an age-dependent decline in sperm quality may influence fertilization success and offspring fitness. It has been hypothesized that DNA damage in the male germline may increase the mutational load carried by an embryo (Radwan, 2003; Johnson & Gemmell, 2012). Velando et al. (2011) showed older male blue-footed booby (*Sula nebouxii*) have higher DNA damage in the germline than middle-aged males (Velando et al., 2011).

Patterns of senescence have primarily been studied across mammals, birds, and fish, while little remains known about amphibians (see Hettyey et al., 2012). In this study, we examined the Mississippi gopher frog as captive populations often experience high rates of reproductive failure (e.g. Richter et al., 2003). Currently, the Mississippi gopher frog is listed as endangered in North America as a result of the extensive destruction of the longleaf pine ecosystem, which the Mississippi gopher frog inhabits (Hammerson et al., 2004a; Lannoo, 2005; USFWS, 2012a). Today, the Mississippi gopher frog is housed at zoological institutions across North America that focus on the recovery (i.e. captive breeding and reintroduction) of this species. In the wild, the Mississippi gopher frog is an explosive breeder; males typically chorus and compete to fertilize eggs deposited on emergent vegetation in shallow ephemeral ponds (Lannoo, 2005). To examine male age in relation to sperm performance that is presumed to affect fertilization success (Edward, 2004; Dziminski et al., 2009), we evaluated different quality-based metrics (motility, concentration, morphology) among three different age
categories that are commonly found in captive zoo populations. Within a zoological setting, it may be beneficial to understand the relationship between male age and sperm performance to better optimize artificial fertilizations by use of male age as a proxy for sperm donors. The aim of our study was to test whether sperm performance declines with male age and to provide a comprehensive investigation to better understand the potential deleterious effects of senescence on reproduction.

Methods

A total of 30 male Mississippi gopher frogs mean ± S.E. snout-vent length = 65.4mm ± 1.18mm (range 52.7 – 75.6 mm), mass = 36.3g ± 1.88g (range 18 – 58 g), age = 3 ± 0.49 years old (range 1-9 years) were used in this study (see Table 2.1 for more details). Animals were either of ‘wild origin’, collected as tadpoles from Glen’s pond in DeSoto National Park (MS, USA) and subsequently captive-reared or were ‘captive-bred’, the first generation of captive-reared frogs having reproductive success in captivity. Animals were housed at either the Detroit Zoo (N=11, all captive-bred; Royal Oak, MI, USA), Memphis Zoo (N=2, wild origin and N=2, captive-bred; Memphis, TN, USA), or Dallas Zoo (N=15, all wild origin; Dallas, TX, USA). All animals were housed in standard plastic polycarbonate tanks or glass tanks fitted with sliding lids. Each fitted with moss and with either a plastic hide or cork bark cave for coverage. Tanks were cleaned once per week, though fresh moss and aged amphibian safe water was added as needed throughout the week (see Table 2.1 for all husbandry details).

Sperm Induction and Collection

To induce spermiation, individuals were given a weight-specific dose of exogenous hormones via intraperitoneal injection (IP) (Poole & Grow, 2012). IP
injections were administered as previous studies have shown IP injections produce increased levels of sperm compared to animals receiving ventral/dorsal absorption or subcutaneous injections (Obringer et al., 2000; Rowson et al., 2001). Immediately following injection, males were placed into separate holding containers filled with approximately 5.0 cm of amphibian safe water to cover the bottom of the container. This allowed frogs to replenish their bladders between collection times. Spermic urine was collected from each male at one-hour post-hormone injection, because a recent study by Watt et al. (2019) showed more sperm was produced at that time point post-injection. Prior to collection, the posterior end of each animal was patted dry using a paper towel to prevent excess water from diluting the sample. Animals were held over a wide petri dish (10 x 1.5 cm) and a piece of catheter tubing (cat#: BB31785-V/5; Scientific Commodities Inc, Lake Havasu City, AZ, USA) was inserted into the cloaca of each male drawing spermic urine into the petri dish. Immediately following urination, the sample was pipetted into a 1.5ml Eppendorf tube (cat#: 05-408-129; FisherScientific, Pittsburgh, PA, USA) and spermic urine volume (µl) in microliters was recorded. Samples were placed in a chilling block (cat#: IC22; Torrey Pines Scientific, Carlsbad, CA, USA) set at 4°C until analysis. All spermic urine samples were analyzed within a five-minute period at each collection time. 

**Sperm Motility**

Within five minutes post-spermic urine collection, sperm were analyzed for each male by pipetting 2µl of spermic urine onto a 2X-CEL glass slide (Hamilton Thorne Biosciences, Beverly, MA, USA), covered with a glass coverslip (22 x 22 mm) and activated with 18µl of 21°C water directly from the male’s enclosure. Video recording
was performed using a CCD B/W video camera module (XC-ST50, Sony, Japan) at 50Hz vertical frequency, mounted on a microscope (CX41 Olympus, Melville, NY, USA), equipped with a 10x negative-phase objective (Byrne et al., 2015; Watt et al. 2019). Percent motility was measured using a generalized progressive motility scale (Kouba et al., 2012; Watt et al. 2019). A total of 100 sperm cells were counted and the number of sperm cells exhibiting motility (sperm with moving flagella that were swimming in a steady forward progression), twitching (sperm with slow-moving flagella with side-to-side head movement), and non-motile sperm (sperm with non-moving flagella with no head movement) were tallied. The percentage of motile sperm was calculated as the number of sperms exhibiting motility out of 100 as counted in all three categories of the progressive motility scale.

**Sperm Concentration**

Sperm concentration was estimated by counting the number of sperm cells in a Neubauer haemocytometer under x400 magnification (Watt et al., 2019). The number of sperm cells in each of the four larger corner squares (1mm²) were counted (64 smaller squares). The mean number of sperm cells in the four larger corner squares was multiplied by the dilution factor. This number was then multiplied by 2500, the standard conversion factor for hemocytometer. Sperm concentration was estimated as the total number of spermatozoa per ml of spermic urine (x10⁶ cells/ml).

**Sperm Morphology**

Immediately following sperm concentration analysis, an aliquot 10µl-20µl was removed from the spermic urine samples and fixed with an equal amount of 8% glutaraldehyde (cat#: G7526-10Ml, Sigm-Adrich, St. Louis, Missouri, USA). Each
sample was stored in an Eppendorf tube and was gently pipetted up and down ten times to ensure proper fixation of spermatozoa present in the sample. Fixed samples were stored in the refrigerator (~ 4°C) until being stained within one-month following fixation. To prepare for staining, sperm samples were gently pipetted ten times using a wide bore transfer pipette to ensure proper mixing of sperm that might have settled. 5µl of each sample was pipetted onto a glass microscope slide (2.5 x 7.5 x 0.1 cm) (cat#: 1301, Globe Scientific Inc., NJ, USA) and was evenly smeared across the surface of the slide. Smeared slides were placed onto a slide warmer (cat#: 3377038, Lab-Line, IA, USA) and left to dry for one hour. Once dried, slides were removed and stained using a Shandon Kwik-Diff Stain Kit (cat#: 9990700, Thermo Scientific, OH, USA). Slides were then placed onto the slide warmer and left for two-hours until dry. Sperm morphology was analyzed using an Olympus BX51 microscope fitted with an Olympus DP72 camera and viewed using a 40x objective lens. Sperm were measured for head length (µm), flagella length (µm), total sperm length (µm) in micrometers using an Olympus DP2-BSW software. Head length (including the midpiece) was measured from the apex of the sperm head to the junction of the flagellum across the midline (Byrne et al., 2003). Flagellum length was measured from the junction of the sperm head to the end of the terminal filament (Byrne et al., 2003). Twenty sperm per male were measured based on a randomization curve showing twenty sperm gave ample accuracy (Watt and Pitcher unpublished data). The mean of the measurements taken on total, head and flagellum length were used in all subsequent analyses.
Statistical Analysis

All data were analyzed using R software v. 2.15.1 (R development Core Team 2012). The effect of male age was examined with respect to motility (%), morphology (µm), concentration (x10^6 cells/ml) and spermic urine volume (µl). To investigate whether each sperm related metrics differed between male age groupings, generalized linear mixed models (GLMM) or linear mixed models (LMM) were used. A GLMM for motility was tested for binomial data (the number of sperm cells that were motile out of 100 sperm cells counted during the progressive motility count were scored as 1, and the remaining sperm out of 100 exhibiting twitching or no motility were scored as 0) with a logit-linked function. LMM’s for concentration, spermic urine volume and sperm morphology were investigated. All GLMM’s used the “glmer” function in the lme4 package in R. All LMM’s used the “lmer” function in the lme4 package in R. For each model, male age categories were the fixed factor, and male ages were binned (1-2, 3-4 and, 8-9 years). Zoo identities (Dallas, Detroit and Memphis) and origin of the animals (‘wild origin’ or ‘captive-bred’) were considered as random factors in the analyses to remove differences in rearing and injection protocols. Age categories were determined according to life history information available for the Mississippi gopher frog. At one to two years of age, male gopher frogs have just come into sexual maturity and have likely undergone their first reproductive event (Richter & Seigel, 2002). Ages 3-4 are middle-aged, and at 8-9 years old, males are at the later end of their natural life expectancy, estimated at 7 years old in the wild. We chose to display data for three different age categories in this analysis to represent the range of male ages used in breeding programs at zoological institutions (Dr. Ruth Marcec, Personal Communication).
We also compared GLMM’s and LMM’s between zoo identities (Dallas, Detroit and Memphis) and origin (‘wild-origin’ or ‘captive-bred’) to account for the possibility that some of the variation may be a result of differences amongst the random factors used in our overall model. Tukey post-hoc analyses were performed to compare differences between zoos and origin. Means are presented as raw means ± standard errors.

**Results**

*Age-Based Model*

There was significant variation in sperm motility with respect to male age groups ($\chi^2 = 145.1, P < 0.001$; Figure 2.1). There were significant differences between age categories (Tukey, $P < 0.05$); however, there was no significant difference in motility between males aged 1-2 years old and males aged 8-9 years old. There was no significant variation on spermic urine volume ($\chi^2 = 0.13, P = 0.72$; Figure 2.2) or sperm concentration ($\chi^2 = 1.07, P = 0.79$; Figure 2.3) between male age categories.

There was significant variation in sperm head length ($\chi^2 = 124.7, P < 0.001$; Figure 2.4), and tukey post-hoc analysis showed there was a significant difference ($P < 0.05$) between all age categories. Tail length was significant with respect to male age groupings ($\chi^2 = 18.6, P < 0.001$; Figure 2.5). There were significant differences between age categories (Tukey, $P < 0.05$); however, there was no significant difference in tail length between males aged 3-4 years old and males 8-9 years old ($P = 0.28$). There was significant variation in sperm total length in relation to age ($\chi^2 = 41.3, P < 0.001$; Figure 2.6), and post-hoc analysis showed there was a difference between all age categories ($P < 0.05$).
Zoo Effects

There was significant variation in sperm motility between the Dallas, Detroit, and Memphis zoos ($\chi^2 = 3.57, P = 0.04$; Figure 2.7). Post-hoc analyses showed there was a significant difference between all zoos ($P<0.05$). There was significant variation in sperm concentration between zoos ($\chi^2 = 7.18, P = 0.002$; Figure 2.8) and post-hoc analyses showed there was a difference between all zoos ($P<0.05$). There was no significant variation on spermic urine volume ($\chi^2 = 2.38, P = 0.07$; Figure 2.9) between zoos.

There was significant variation in sperm head length among zoos ($\chi^2 = 230.42, P<0.001$; Figure 2.10), and post-hoc analyses showed there was a significant difference in sperm head length between all zoos ($P<0.05$). Tail length was significant among zoos ($\chi^2 = 74.06, P < 0.001$; Figure 2.11) and post-hoc analyses showed there was a difference in tail length between the Dallas Zoo and the Detroit and Memphis Zoo ($P<0.05$). There was significant variation in sperm total length among zoos ($\chi^2 = 126.5, P < 0.001$; Figure 2.12), and post-hoc analysis showed there was a difference between the Dallas Zoo and the Detroit and Memphis Zoo ($P<0.05$).

Origin Effects

There was significant variation in sperm concentration based on a male’s origin (‘wild-origin’ or ‘captive-bred’) ($\chi^2 = 5.71, P = 0.02$; Figure 2.13). Captive-bred males were found to have significantly higher sperm concentration. There was significant variation in sperm head length ($\chi^2 = 89.71, P < 0.001$; Figure 2.14), tail length ($\chi^2 = 85.64, P < 0.001$; Figure 2.15), and total length ($\chi^2 = 112.3, P < 0.001$; Figure 2.16). For head, tail, and total length, captive-bred males had a significantly longer length compared
to wild-origin males. There was no significant variation on sperm motility ($\chi^2 = 2.67, P = 0.11$; Figure 2.17) or spermic urine volume ($\chi^2 = 2.41, P = 0.06$; Figure 2.18).

**Discussion**

In this study we examined sperm quality metrics between three different age categories (typical of those found in captivity) focused on an endangered species of frog in order to better understand the potential deleterious effects of senescence on reproduction and if so, to make this data available in order to increase the efficacy of captive breeding efforts in zoos for reintroduction. Our results demonstrate that male age has a significant effect on several sperm performance metrics in the Mississippi gopher frog.

We found that sperm morphology metrics are positively related to male age, including head length, tail length, and total sperm length. Uniquely, our study showed that as males age their sperm elongate, and males aged 8-9 years have the longest total sperm length (~15%) compared to 1-2 years old and 3-4 years old males. This result runs contrary to life history theory in the context of energy allocation, as males should produce numerous, small sperm to optimize their reproductive output (Green, 2003). In support of our finding, in rove beetles (*Aleochara bilineata*), old males have been shown to produce significantly longer sperm (Green, 2003). One possible explanation for our observations may be that older males shift their life history ‘optimization’ to reproduction in later years. In captivity, husbandry drastically minimizes environmental constraints, modifying the ‘natural’ energetic tradeoffs between growth, reproduction, and maintenance (Heath et al., 2003). Therefore, older males may possess the necessary resources to produce sperm of higher quality, whereas younger males still need to
allocate a higher proportion of their resources to growth and maintenance. This is especially the case for juveniles experiencing their first reproductive event (Green, 2003; Folkvord et al., 2014). It may also be possible that longer sperm were produced by males with absolutely larger testes. Sperm length may be an adaptive trait under sperm competition and over time a causal relationship between sperm production and testes size may occur. Similar findings have been reported in amphibians (Jennions & Passmore, 1993; Byrne et al., 2002), although we did not evaluate this trait in our study due to the rarity of the Mississippi gopher frog. However, to ensure that sperm length was not simply a by-product of body condition, we performed additional statistical analyses and found no significant variation amongst age categories. We also found there was no significant difference between sperm concentration or spermic urine volume between male age categories.

Motility was found to be highest amongst middle-aged males. While largely untested in amphibians (but see Hettyey et al., 2012), this result seems to contrast the majority of studies in non-human taxa that have found there to be no effect of age on sperm motility and velocity (reviewed in Johnson & Gemmell, 2012). However, our findings are consistent with the notion that an age-related decline in motility may be an outcome of senescent sperm performance (Radwan, 2003). Anuran sperm remain immotile in the testis until exposed to a hypotonic environment when the decrease in osmolality activates motility (O'Brien et al., 2011). Energy metabolism for sustaining motility is dependent on the number of mitochondria available to produce ATP for proper sperm function (Burness et al., 2004; Brown et al., 2015). In older males, age-related declines in motility have been linked to oxidative stress, where the production of reactive
oxygen species (ROS) accumulate from high metabolic activity (Siva-Jothy, 2000; Koppers et al., 2008). This can cause defective sperm function and potentially impede fertilization caused by damage to the sperm’s plasma membrane (Aitken et al., 2014). Oxidative stress is a key metabolic mechanism underlying life-history trade-offs in animals, such that animals with a higher metabolic rate, produce higher ROS (Selman et al., 2012). Supporting this, in the Brown Norway rat, sperm from older males was more susceptible to oxidative stress than younger males, which resulted in ROS-induced damage to the sperm’s membrane (Zubkova & Robaire, 2006; Weir & Robaire, 2007). As sperm production is costly, an increased ROS-induced oxidative stress at the organismal level may indicate why we observed a decline in motility amongst males aged 8-9. By contrast, younger males may simply have a lower motility as a result of the limited energetic thresholds available for ATP storage (Sasson et al., 2012; Brown et al., 2015). Such an outcome may be important, as sperm motility is considered to be a key determinant of fertilization success in anurans (Dziminski et al., 2009). In anurans, sperm must remain motile long enough to locate and pass through the jelly layers that surround a fertile oocyte (O’Brien et al., 2011). This notion was supported by Dziminski et al. (2009) that showed males with a higher proportion of motile spermatozoa had a greater fertilization advantage.

Having tested that sperm quality would differ between male age categories, we wanted to further delineate the possibility that some of the variation may be a result of the difference amongst the three zoos used in this study that differ to some extent in their husbandry and captive breeding techniques (see Table 2.1). Male Mississippi gopher frogs housed at the three zoological institutions differed in environmental cues and
induction protocols. Across all morphology metrics, motility, and concentration, zoo identity was found to have a significant effect. First, housing conditions at each zoo that provided us samples differed as animals at the Dallas zoo were housed in solitary conditions, while animals at the Detroit and Memphis Zoo were housed in mixed-sex groups (male-female or male-male). It has been shown that an adaptive plasticity or a ‘priming effect’ can influence the quality of sperm, occurring when males are housed in a competitive environment (Gasparini et al., 2009). For example, in the guppy (*Poecilia reticulata*) sperm velocity was shown to increase when males were in the presence of females (Gasparini et al., 2009). Similarly, in the fowl (*Gallus gallus*) sperm speed increased in the presence of higher quality females (Cornwallis & Birkhead, 2007).

Interestingly, across most traits measured (see Table 2.2), there was no significant difference between sperm performance in males housed in mixed sex groups, though they were notably higher than males housed in solitary. Secondly, in captivity, the Mississippi gopher frog has not been observed to breed naturally and requires assisted reproductive technologies, such as exogenous hormones to reproduce (Poole & Grow, 2012). In captive breeding programs, both luteinizing hormone-releasing hormone (LHRH) and human chorionic gonadotrophin (hCG) are commonly used to stimulate gamete production (Kouba et al., 2012). In this study, the Dallas and Memphis Zoo used a hormone cocktail of both exogenous hormones for induction, while the Detroit Zoo used LHRH only. Differences in spermiation response and sperm quality tend to be species specific and dependent on the type of hormone administered (Kouba et al., 2012). For example, in the American toad (*Anaxyrus americanus*) hCG was found to produce a higher sperm concentration than LHRH (Kouba et al., 2012). However, there was no
significant difference found in sperm motility between hCG or LHRH. Using this example, we may be able to draw a parallel, as males at the Memphis zoo produced approximately 73% more spermatozoa than males at the Detroit zoo. Similarly, no difference in sperm motility was found between the Detroit Zoo and the Memphis or Dallas Zoo, independent of the exogenous hormone used.

Furthermore, we wanted to assess the potential variation that may have resulted from the difference in origin amongst the males used in this study that differ as being of ‘wild origin’, or ‘captive-bred’ (see Methods). Across all metrics found to be significant (see Table 2.3), ‘captive-bred’ males had the highest sperm performance. In both wild and captive environments, anuran reproductive behavior can be influenced by environmental stimuli (Tsai, 2011). Here, one possibility for our observations may be tied to an enriched environment provided by captivity. Zoos employ a high quality of care, such as controlling environmental stimuli (i.e. temperature, photoperiod, etc.) and nutrition, by gut-loading insects with vitamins and minerals (Poole & Grow, 2012). Here, the benefits of captivity may simply improve an animal’s wellbeing and thus, their reproductive function (Kouba et al., 2012a).

Finally, this study has demonstrated that male age has a significant effect on several sperm performance metrics in the Mississippi gopher frog. To investigate the possibility that a decline in sperm quality may negatively impact fertilization success and offspring fitness, we conducted a brief pilot study (see Appendices for details). Using a split-clutch in-vitro fertilization design we tested the effects of age on fertilization and hatching success using relatively young males (i.e. 1-2, 3-4 years old) and relatively old males (i.e. 3-4, 7-9 years old). Our results suggest there is a positive association between
fertilization success and older males. In the future, we plan on continuing our investigation to better understand the relationship between male age and fertilization success and offspring fitness.

Our research highlights the need to investigate male sperm performance across amphibian species to better understand reproductive success in the context of ageing and senescence theory. By providing a comprehensive overview of age categories spanning the gopher frog’s lifespan, our data is relevant for zoological breeding programs. In conclusion, the Mississippi gopher frog typically lives 10 years when maintained in captivity. Our study indicates that males retain their reproductive capacity for most of their lifespan. However, as space in captive breeding facilities is limited and the need for maximal productivity is essential to produce offspring to supply the reintroduction program, we suggest, males aged 3-4 years and 8-9 years may be optimal to breed as they show the highest sperm performance.
References


Johnson SL, Gemmell NJ. 2012. Are old males still good males and can females tell the difference? Bioessays 34(7),609-619.


Amphibian Husbandry Resource Guide. 2.0 ed. Silver Spring, MD: Association of Zoos & Aquariums.


Table 2.1 Mean ± S.E. snout-vent length (mm), mass (g), and age (years) for male Mississippi gopher frogs across zoos. Table shows summary of lighting (daily photoperiod), enclosure design (glass vs. plastic, and size), coverage (enrichments), substrate type (shag or sphagnum moss), water quality, and food type provided to gopher frogs used in this study between zoos. Exogenous hormone dosage and type (hCG or LHRH) are reported between zoos.

<table>
<thead>
<tr>
<th></th>
<th>Dallas Zoo</th>
<th>Detroit Zoo</th>
<th>Memphis Zoo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size (n)</td>
<td>14</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Snout-vent length (mm)</td>
<td>63.48 ± 1.60</td>
<td>66.33 ± 2.22</td>
<td>69 ± 1.97</td>
</tr>
<tr>
<td>Mass (g)</td>
<td>34.44 ± 2.05</td>
<td>37.82 ± 4.17</td>
<td>38 ± 3.87</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.64 ± 0.13</td>
<td>8.33 ± 1.02</td>
<td>2.8 ± 1.32</td>
</tr>
<tr>
<td>Lighting</td>
<td>8am – 6pm daily</td>
<td>7am – 8pm daily</td>
<td>8am – 6:30pm daily</td>
</tr>
<tr>
<td>Enclosure</td>
<td>Glass Aquaria (50.8 cm x 25.4 cm x 30.5 cm)</td>
<td>Plastic polycarbonate tanks (55.8 cm x 38.1 cm x 35.56 cm)</td>
<td>Glass Aquaria (50.8 cm x 25.4 cm x 30.5 cm)</td>
</tr>
<tr>
<td>Coverage</td>
<td>Plastic hid</td>
<td>Plastic hid or cork bark</td>
<td>-</td>
</tr>
<tr>
<td>Substrate Type</td>
<td>Sphagnum moss</td>
<td>Shag moss</td>
<td>Sphagnum moss</td>
</tr>
<tr>
<td>Water Quality</td>
<td>Aged amphibian safe water</td>
<td>Aged amphibian safe water</td>
<td>Aged amphibian safe water</td>
</tr>
<tr>
<td>Food Type</td>
<td>Gut loaded crickets – ad libitum</td>
<td>Gut loaded crickets, Dubia roaches, soldier flies (2x/week), mealworms and wax worms with Repashy supplement and all feed was dusted with Nekton vitamin supplement</td>
<td>Gut loaded crickets – ad libitum</td>
</tr>
<tr>
<td>Human Chorionic Gonadotrophin (hCG)</td>
<td>10IU/g</td>
<td>-</td>
<td>10IU/g</td>
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<tr>
<td>Luteinizing hormone releasing hormone (LHRH)</td>
<td>0.4μg/ BW</td>
<td>0.5μg/BW</td>
<td>0.4μg/ BW</td>
</tr>
</tbody>
</table>
Table 2.2 Summary table of sperm performance metrics analyzed using a generalized linear mixed model or linear mixed model to test zoo effects. Chi-square and P-value are the same as in results. Zoo effects state which zoo had the best sperm performance for that given metric.

<table>
<thead>
<tr>
<th>Sperm Metric</th>
<th>$\chi^2$</th>
<th>P</th>
<th>Zoo Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>3.57</td>
<td>0.04</td>
<td>Memphis</td>
</tr>
<tr>
<td>Concentration</td>
<td>7.18</td>
<td>0.002</td>
<td>Memphis</td>
</tr>
<tr>
<td>Spermic Urine</td>
<td>2.38</td>
<td>0.07</td>
<td>-</td>
</tr>
<tr>
<td>Head Length</td>
<td>230.42</td>
<td>&lt;0.001</td>
<td>Memphis</td>
</tr>
<tr>
<td>Tail Length</td>
<td>74.06</td>
<td>&lt;0.001</td>
<td>Detroit/Memphis</td>
</tr>
<tr>
<td>Total Length</td>
<td>126.5</td>
<td>&lt;0.001</td>
<td>Detroit/Memphis</td>
</tr>
</tbody>
</table>
Table 2.3 Summary table of sperm performance metrics analyzed using a generalized linear mixed model or linear mixed model to test origin effects. Chi-square and P-value are the same as in results. Origin effects state which origin had the best sperm performance for that given metric.

<table>
<thead>
<tr>
<th>Sperm Metric</th>
<th>$\chi^2$</th>
<th>P</th>
<th>Origin Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>2.67</td>
<td>0.11</td>
<td>-</td>
</tr>
<tr>
<td>Concentration</td>
<td>5.71</td>
<td>0.02</td>
<td>Captive-bred</td>
</tr>
<tr>
<td>Spermic Urine</td>
<td>2.41</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>Head Length</td>
<td>89.71</td>
<td>&lt;0.001</td>
<td>Captive-bred</td>
</tr>
<tr>
<td>Tail Length</td>
<td>85.64</td>
<td>&lt;0.001</td>
<td>Captive-bred</td>
</tr>
<tr>
<td>Total Length</td>
<td>112.3</td>
<td>&lt;0.001</td>
<td>Captive-bred</td>
</tr>
</tbody>
</table>
Figure 2.1  Raw means (± 1 SE) for motility (%) across male’s age groups (years) in the Mississippi gopher frog (*Lithobates sevosus*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.2  Raw means (± 1 SE) for spermic urine volume (µl) across male’s age groups (years) in the Mississippi gopher frog (*Lithobates sevosus*).
Figure 2.3  Raw means (± 1 SE) for sperm concentration (x10⁶ cells/ml) across male’s age groups (years) in the Mississippi gopher frog (*Lithobates sevosus*).
Figure 2.4  Raw means (± 1 SE) for sperm head length (µm) across male’s age groups (years) in the Mississippi gopher frog (Lithobates sevosus). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.5  Raw means (± 1 SE) for sperm tail length (µm) across male’s age groups (years) in the Mississippi gopher frog (*Lithobates sevosus*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.6  Raw means (± 1 SE) for sperm total length (µm) across male’s age groups (years) in the Mississippi gopher frog (*Lithobates sevosus*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
**Figure 2.7** Raw means (± 1 SE) for motility (%) between zoos (Dallas, Detroit, Memphis) in the Mississippi gopher frog (*Lithobates sevosus*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.8  Raw means (± 1 SE) for concentration (x10^6 cells/ml) between zoos (Dallas, Detroit, Memphis) in the Mississippi gopher frog (*Lithobates sevius*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.9  Raw means (± 1 SE) for spermic urine volume (µl) between zoos (Dallas, Detroit, Memphis) in the Mississippi gopher frog (*Lithobates sevosus*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.10  Raw means (± 1 SE) for sperm head length (µm) between zoos (Dallas, Detroit, Memphis) in the Mississippi gopher frog (*Lithobates sevosus*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.11 Raw means (± 1 SE) for sperm tail length (µm) between zoos (Dallas, Detroit, Memphis) in the Mississippi gopher frog (*Lithobates sevosus*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.12  Raw means (± 1 SE) for sperm total length (µm) between zoos (Dallas, Detroit, Memphis) in the Mississippi gopher frog (*Lithobates sevosus*). Shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 2.13  Raw means (± 1 SE) for sperm concentration (x10^6 cells/ml) between origin ('wild-origin', 'captive-bred') in the Mississippi gopher frog (*Lithobates sevosus*).
Figure 2.14  Raw means (± 1 SE) for sperm head length (µm) between origin ('wild-origin', 'captive-bred') in the Mississippi gopher frog (*Lithobates sevius*).
Figure 2.15  Raw means (± 1 SE) for sperm tail length (µm) between origin (‘wild-origin’, ‘captive-bred’) in the Mississippi gopher frog (*Lithobates sevosus*).
Figure 2.16  Raw means (± 1 SE) for sperm total length (µm) between origin (‘wild-origin’, ‘captive-bred’) in the Mississippi gopher frog (*Lithobates sevosus*).
Figure 2.17 Raw means (± 1 SE) for motility (%) between origin (‘wild-origin’, ‘captive-bred’) in the Mississippi gopher frog (*Lithobates sevosus*).
Figure 2.18  Raw means (± 1 SE) for spermic urine volume (µl) between origin (‘wild-origin’, ‘captive-bred’) in the Mississippi gopher frog (*Lithobates sevosus*).
CHAPTER 3
TIME FROM INJECTION OF LUTEINIZING HORMONE RELEASING HORMONE AFFECTS SPERM QUALITY IN THE CRITICALLY ENDANGERED MISSISSIPPI GOPHER FROG (*Lithobates sevosus*)

Introduction

Historically, the Mississippi gopher frog, (*Lithobates sevosus*) has been found along the southern Gulf Coastal Plain of Louisiana, Mississippi, and Alabama (Hammerson, Richter, Siegel, LaClaire, & Mann, 2004a). Accelerated declines in the number of viable populations have been observed over the past century. These declines are primarily due to urban sprawl and the destruction of the longleaf pine ecosystem upon which the Mississippi gopher frog is reliant on for reproductive success. The extensive destruction of the ecosystem has resulted in the disappearance of animals from Alabama since 1922 and Louisiana since 1965 (Hammerson et al., 2004a; Lannoo, 2005). By 2012, the Mississippi gopher frog was listed as critically endangered and only two populations were known to exist in Harrison and Jackson Counties, Mississippi, with an estimated 100 individuals (Hammerson et al., 2004a). Concerned for the future of the Mississippi gopher frog, the United States Fish and Wildlife Service (USFWS) established partnerships with a variety of zoological institutions dedicated to the recovery of this species (Richter, Crother, & Broughton, 2009). Today, the Mississippi gopher frog has a species survival plan (SSP), which is a program developed by the Association of Zoos and Aquariums (AZA), that ensures the survival and recovery of endangered species (Conway, 2011). The SSP oversees the Mississippi gopher frog recovery plan that is
currently focused on captive breeding and the reintroduction of froglets into their historic range.

One of the SSP’s main concerns for the future success of the Mississippi gopher frog are the challenges faced in captive breeding. Captive populations often experience high rates of reproductive failure (e.g. Richter, Young, Johnson, & Seigel, 2003). The exact cause of low reproductive success is unknown; though it is suspected that it is due to an inability to replicate the natural environmental cues that lead to a reproductive event (Kouba, Vance, & Willis, 2009). Reproductive failure or dysfunction can occur in both sexes, though in males, a lack of breeding behaviors, such as amplexus or calling is common. Reproductive dysfunction can also result in the failure to produce sperm, requiring exogenous hormones to induce spermiation; the process by which mature spermatids are released from the supporting somatic Sertoli cells into the lumen of the seminiferous tubule (O’Donnell, Nicholls, O’Brien, McLachlan, & Stanton, 2011). As breeding efforts have become increasingly relevant for zoological institutions, it is therefore imperative to investigate hormonal induction and gamete quality for the success of this species.

For half a century, exogenous hormones have been used in captive breeding programs to induce a spermiation response in a variety of frog and toad species (Kouba, delBarco-Trillo, Vance, Milam, & Carr, 2012). In anurans, both luteinizing hormone-releasing hormone analog (LHRHa) and human gonadotropin (hCG) are commonly used to stimulate gamete production (Goncharov, Shubrayy, Serinova, & Uteshev, 1989; Roth & Obringer, 2003; Kouba et al., 2012a). Primarily, studies have focused on identifying the range of hormone concentration required to initiate a spermiation response, which has
been found to vary widely between species (Kouba et al., 2012a; Kouba et al., 2012). Although several reviews exist, the response time and quality of sperm production remains largely unknown for many endangered species. Identifying this information is critical for zoos that regularly employ exogenous hormones to successfully breed these animals in captivity.

Sperm quality measures, including motility, velocity, and concentration are major determinants of fertilization success (Dziminkski, Roberts, Beveridge, & Simmons, 2009; Johnson, Butts, Wilson, & Pitcher, 2013). For example, in the spotted grass frog (*Limnodynastes tasmaniensis*) sperm concentration was found to have a significant effect on fertilization rate when sperm concentration was greater than $10^4$ sperm/ml (Edwards, Mahony, & Clulow, 2004). Essentially, these measures of sperm quality can be used to optimize captive breeding protocols, which have become critical to captive facilities that often experience high reproductive failure (Kouba et al., 2012). Sperm quality has been found to be highly dependent on sampling time post-hormone injection (Obringer et al., 2000; Byrne & Silla, 2010; Tonga et al., 2017). For example, in the critically endangered Panamanian golden frog (*Atelopus zeteki*) sperm concentration, percentage of motile sperm cells, and morphology were found to vary significantly across sampling time and hormone dosage (Tonga et al., 2017). Further research evaluating the spermiation response post-hormone injection, however, focuses primarily on sperm concentration in response to hormone dosage or type (Obringer et al., 2000; Byrne & Silla, 2010; Kouba et al., 2012; Tonga et al., 2017). It is therefore imperative to fully characterize sperm quality (i.e. motility, velocity, and concentration) from time post hormone-injection to optimize reproductive protocols for higher fertilization success.
In our study, we examined three time points post-LHRHa injection to evaluate sperm quality in the Mississippi gopher frog. These time points were chosen to decrease stress on the animals and to allow time for spermic urine to build between the collection times. Understanding sampling time in sperm quality can be used to optimize fertilization success for endangered species of true frogs housed at zoological institutions and can increase the efficiency of captive breeding programs.

Methods

A total of 11 male Mississippi gopher frogs (*Lithobates sevosus*) mean ± S.E. snout-vent length = 66.68mm ± 2.05mm (range 52.7 – 75.6 mm), mass = 37.66g ± 3.81g (range 18 – 58 g), age: 5.55 ± 3.59 years old (range 1-9 years) housed at the National Amphibian Conservation Center (Detroit Zoo, Royal Oak, MI, USA) were used in this study. All animals at the Detroit Zoo were kept on a natural light cycle operated by a timer which turned on at 7am and turned off at 8pm daily. Housing conditions consisted of standard plastic polycarbonate tanks (4,620 inches³) fitted with sliding lids. Each lid was cut on the inside perimeter to allow light to penetrate the tank. Lighting was provided by EIKO track light bulbs that were modified with removed glass to allow UV to access each tank. Approximately half of each tank bottom covered with shag moss and cork bark and all tanks were fitted with either a plastic hide or a cork bark cave for coverage. Tanks were tilted at a 30° angle and filled with 21°C aged amphibian safe water, at approximately 7.62cm depth to create a pond at the front of the tank. Tanks were cleaned once per week, though fresh moss and water were provided as needed throughout the week. Adult Mississippi gopher frogs were provided prey items (gutload crickets (*Gryllidae*), Dubia roaches (*Blaptica dubia*), soldier flies (*Stratiomyidae*)) twice a week.
Mealworms (*Tenebrio molitor*) and wax worms (*Pyralidae*) were gut loaded prior to feeding using Repashy supplement and all feed was dusted with Nekton vitamin supplement.

**Hormone Treatment**

Male Mississippi gopher frogs were given weight specific doses of exogenous hormone to produce spermic urine (see Poole & Grow, 2012). Each male received an intraperitoneal injection of 0.5µg/g body weight of a luteinizing hormone-releasing hormone analog (cat#: L4513; Sigma-Aldrich, St. Louis, Missouri, USA).

**Sperm Sampling**

Immediately following hormone injection, male frogs were placed into holding containers fitted with shag moss. Each container was filled with approximately 2.54 cm of amphibian safe water to cover the bottom of the container. This allowed frogs to replenish their bladders between collection times. Spermic urine samples were collected at three time points: 30 minutes, 60 minutes and 120 minutes post-injection. Spermic urine was also collected at time zero to make sure there was no sperm present. Prior to collecting urine, the posterior end of each animal was patted dry using a paper towel to prevent excess water from diluting the sample. Animals were held over a wide petri dish (1808.48 cm³) and a soft piece of catheter tubing (#BB31785-V/5; Scientific Commodities Inc, Lake Havasu City, AZ) was inserted into the cloaca of each male drawing spermic urine into the petri dish. Immediately following urination, the sample was pipetted into a 1.5mL Eppendorf tube (#05-408-129; FisherScientific, Pittsburgh, PA) and placed in a chilling block (#IC22; Torrey Pines Scientific, Carlsbad, CA) set at 4°C until sperm analysis could take place (see below). All spermic urine samples were
analyzed within a five-minute period at each collection time to avoid artifacts caused by a
time difference between analyses.

*Sperm Quality*

Up to five minutes post spermic urine collection, sperm were recorded at three
different sampling times (30mins, 60mins, 120mins post-injection) for each male by
pipetting 2µl of spermic urine onto a 2X-CEL glass slide (Hamilton Thorne Biosciences,
Beverly, MA, USA), covered with a glass coverslip (22 x 22 mm) and activated with
18µl of 21°C water directly from the male’s enclosure. Sperm were recorded using a
CCD B/W video camera module (XC-ST50, Sony, Japan) at 50Hz vertical frequency,
mounted on a microscope (CX41 Olympus, Melville, NY, USA), equipped with a 10x
negative-phase objective. Videos were converted into uncompressed AVI files using
VirtualDubMod 1.5.10.2 (https://virtualdubmod.en.uptodown.com/windows), an open
source video capture and processing tool. Videos were analyzed using a java-based image
processing program, ImageJ (Schneider, Rasband, & Eliceiri, 2012). To set a fixed scale,
one video was selected at random and a still image was captured and opened into a
Microsoft word document (Version 15.40). In Microsoft word, gridlines were overlaid
across the image and set to 1mm x 1mm. The altered image was uploaded to ImageJ, and
a fixed scale was set by clicking analyze → set scale → 1mm. For each video, sperm
straight line velocity (µm/sec) was analyzed at one-minute post-activation. Sperm
velocity was calculated in microns/second based on the time-average velocity of a sperm
head along the straight line between its first and last detected position. Sperm motility
and progressive motility were measured using a progressive motility scale (Kouba et al.,
2012). Before analysis, samples were gently pipetted several times using a wide bore
transfer pipette. For each male, 2µl of spermic urine was pipetted onto a plain glass microscope slide (#12-550-A3; Fischer Scientific, Hampton, NH), covered with a glass coverslip (22 x 22 mm) and activated with 18µl of 21°C water directly from the male’s enclosure. A total of 100 sperm cells were counted and the number of cells exhibiting progressive motility (sperm with rapidly moving flagella in a steady forward progression), motility (sperm with moving flagella that were swimming in a steady forward progression), twitching (sperm with slow-moving flagella with side to side head movement), and non-motile sperm (sperm with non-moving flagella with no head movement) were tallied. The percentage of progressively motile and motile sperm were calculated as the number of sperms exhibiting progressive motility or motility out of 100 as counted in all categories of the progressive motility scale.

_Sperm Concentration_

Sperm concentration was estimated by adding 10µl of spermic urine to 190µl of amphibian safe water. Each aliquot was gently pipetted using a wide-bore transfer pipette, and 10µl was placed onto a Neubauer haemocytometer under x400 magnification. Sperm cells were counted in 5 squares (1mm²), 4 corner squares and the center square. Concentration was estimated by counting the mean number of cells per square count (i.e. mean of the five squares) for the two sides of the haemocytometer. The mean number was multiplied by 25 and then by 10 (chamber depth in µm) (Pitcher, Doucet, Beausoleil, & Hanley, 2009). This number was then multiplied by the initial volume of the sample divided by the volume of the original mixture in the sample. Sperm concentration was estimated as the total number of sperms per ml of spermic urine (x10⁶ cells/ml).
Statistical Analysis

To examine the effect of time post-hormone injection on sperm quality metrics in the Mississippi gopher frog, two statistical approaches were used. Time post-hormone injection was examined with respect to motility (%), progressive motility (%), velocity (µm/sec), and concentration (x10^6/ml) by fitting quadratic equations to the data. The most common pattern of post-hormone injection sampling time in sperm quality across species is quadratic. Quadratic patterns generally represent a bell-shaped curve in which sperm quality increases at a sampling time post-hormone injection, peaks at an optimal sampling time post-hormone injection, and then decreases at a sampling time post-hormone injection (e.g. Kouba et al., 2012). By fitting quadratic equations to the data, the potential positive or negative relationship between post-hormone injection time points could be explored. The second approach analyzed all of the data per sperm quality metric over all three of the sampling time points using a repeated measures mixed-model ANOVA. This approach was able to examine whether there were significant differences in sperm quality between the three sampling times post-injection (30min, 60mins, 120mins) at one-minute post-activation. This time point post-activation was chosen as an arbitrary value prior to the egg’s jelly coat hardening, which occurs approximately five minutes post-egg release (Poole & Grow, 2012). Sampling time post-injection was considered a fixed factor, whereas male identity and male age were considered as random factors. Akaike’s (AIC) and Bayesian (BIC) information criteria were used to assess which model was most appropriate. Tukey post-hoc analyses were used to compare means between times post-injection. All data was analyzed using R, a programming language for statistical computing (Version 3.5.1; package lsmeans, package lme4).
Results

Motility

There was no significant quadratic relationship between motility and sampling time post-activation found ($r^2 = 0.12; F_{2,30} = 2.01, P = 0.15, y = 20.55x - 15.27x^2 - 37.82$). The second approach showed post-injection sampling time has a significant effect on sperm motility post-activation ($F_{2,20} = 6.84; P = 0.005$; Figure 3.1).

Progressive Motility

No significant quadratic relationship between progressive motility and sampling time post-activation was found ($r^2 = 0.007; F_{2,30} = 0.11, P = 0.9, y = -0.18x + 0.36x^2 + 3.36$). Sampling time had no significant effect on progressive motility post-activation ($F_{2,20} = 6.79; P = 0.86$; Figure 3.2).

Velocity

No significant quadratic relationship between velocity and sampling time post-activation was found ($r^2 = 0.028; F_{2,30} = 0.84, P = 0.44, y = 14.51x - 5.75x^2 + 5.50$). The second approach showed post-injection sampling time had a significant effect on velocity post-activation ($F_{2,20} = 3.80; P = 0.03$; Figure 3.3).

Concentration

There was no significant quadratic relationship between concentration and sampling time post-activation found ($r^2 = 0.15; F_{2,30} = 2.62, P = 0.09, y = 150.14x - 65.30x^2 - 28.47$). Sampling time had a marginally significant effect on sperm concentration ($F_{2,20} = 3.45; P = 0.05$; Figure 3.4).
Discussion

Here, we provide viability of sperm quality metrics key for successful artificial propagation (i.e. motility, velocity, and concentration) at three times post-injection. This study was designed to enhance our understanding of amphibian induction using LHRHa to increase the efficiency of captive breeding programs. Our results demonstrate that sampling time post-injection has a significant effect on sperm quality metrics in the Mississippi gopher frog. Time since hormone injection in sperm quality significantly affected percent motility and velocity (p < 0.05) and had a marginal effect on sperm concentration (p = 0.05). However, time since hormone injection had no specific effect on progressive motility (p > 0.05). These results have important implications for optimizing fertilization success for endangered species of imperiled frogs in captive breeding programs.

Consistent with studies on other species of endangered anurans, our results suggest that percent motility can be affected, and concentration may be affected by how long after injection time you collect the sperm sample. Variation in sperm quality across sampling time has been previously reported for a number of endangered frog and toad species (Obringer et al., 2000; Byrne & Silla, 2010; Togna et al., 2017). For example, Obringer et al. (2000) assessed the spermiation and sperm quality (i.e. motility and concentration) related to several methods of LHRH administration (intraperitoneal injection, subcutaneous injection, ventral dermal absorption, and dorsal dermal absorption) and dosage levels (1.0µg, 0.1µg, and 0.01µg) in the American toad (Bufo americanus). Peak sperm concentration was found to differ between type of hormone administration and dosage across time in the American toad. Across all sampling times
(0h, 3h, 7h, and 12h) post-LHRH injection, intraperitoneal injected males reached a maximum sperm production earlier than subcutaneous injected males, with peak sperm concentration occurring 12h post-injection (1.0µg dosage). Previous research from Togna et al. (2017) also evaluated post-injection sampling time to analyze the concentration of spermatozoa in the critically endangered Panamanian golden frog following different hormone inductions. Results showed that sampling time had a significant effect on sperm concentration, with the peak sperm concentration occurring between 2.5 to 4.5 h post-hormone injection. Here, it is important to recognize differences in sperm metrics (i.e. concentration) between endangered anurans. For example, very high concentrations ($10^4$/mL to $10^6$/mL) of anuran sperm can increase fertilization rates during in-vitro fertilization (Browne et al. 2015). Thus, determining the peak sampling time post-injection can provide a significant advantage for maximizing reproductive success in captivity.

In our study, sperm straight line velocity (VSL) was found to be significantly affected by sampling time post-injection. However, this metric has not previously been studied in anurans in the context of sampling time. Quantifying VSL can be useful for estimating fertilization success as frog sperm are structurally and behaviourally different from the sperm of other external fertilizers (Hettyey & Roberts, 2006; Dziminkski et al., 2009). For example, Dziminkski et al. (2009) found that males with slower swimming velocities have an advantage in competitive fertilization. Our evaluation of sperm velocity can act as a starting place to better understand how velocity is influenced by sampling time. Future studies would likely benefit from using velocity as a metric to quantify fertilization success in-vitro.
Conflicting with other studies on anurans, progressive motility was not significantly affected by sampling time. Browne et al. (2006) found progressive motility differed across sampling time and hormone administration in the endangered Wyoming toad, *Bufo baxteri*. Progressive motility (22%) was found to be low at 3h post-human chorionic gonadotropin (hCG) injection, before reaching a maximum motility (95%) at 5h post-hCG injection. Similar results were found by Kouba et al. (2012), showing that hCG significantly induced an effective spermiation response over sampling time than LHRHa in the American toad, *Anaxyrus americanus*. The effect found with hCG suggests that other hormone types may be a valid alternative to LHRHa. Our study, however, was limited to the use of LHRHa, a hormone commonly used at zoological institutions. Future studies on the Mississippi gopher frog’s spermiation response to different hormone treatments may be optimal to better breeding protocols.

In conclusion, our results suggest that sampling time post-injection by LHRHa can have a significant impact on the quality of sperm motility and velocity, while having a marginal effect on concentration, and no significant effect on progressive motility. Understanding the spermiation response to LHRHa for Mississippi gopher frogs is key to maximizing reproductive success in captive breeding programs. Globally, anuran populations are in great decline, demonstrating the importance of enhancing breeding protocols in zoological institutions that are active in *in-situ* and *ex-situ* conservation. Overall, these results could prove useful for maximizing fertilization success if sperm is sampled at optimal times post-hormone injection.
References


Figure 3.1 Raw means for motility (%) across post-injection sampling time (minutes).

Means (± 1 SE) with shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 3.2 Raw means for progressive motility (%) across post-injection sampling time (mins). Means (± 1 SE) with shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
Figure 3.3 Raw means for velocity (µm/sec) across post-injection sampling time (mins).

Means (± 1 SE) with shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
**Figure 3.4** Raw means for concentration (x10^6 cells/ml) across post-injection sampling time (mins). Means (± 1 SE) with shared letters did not differ significantly from one another based on Tukey post-hoc analyses.
SUMMARY

The Mississippi gopher frog (*Lithobates sevosus*) is an example of an endangered species of frog which is currently undergoing captive breeding efforts across zoos in the United States. These efforts are being impaired by the reproductive dysfunction experienced by the captive populations which may be potentially due to ageing effects and low-quality gamete expression. The objective of this thesis was to assess the effect of male age on sperm quality and investigate the use of an assisted reproductive technology (i.e. LHRHa hormone injection) to characterize sperm quality across sampling time to improve the efficacy of breeding protocols. This chapter provides a summary of the key findings in Chapter 2 and Chapter 3 and will provide future directions on ageing and hormone induction in the Mississippi gopher frog.

Chapter 2

In captive breeding programs, reproductive dysfunction may be due to ageing effects resulting in low-quality gamete expression (Poole & Grow, 2012). While ageing effects have been studied across a variety of non-human taxa, there is little known on amphibians. In Chapter 2, I investigated the variation in sperm quality between males categorized into three age groupings. Different aged males across the species expectant lifespan (1-9 years) were chosen to identify an optimal breeding age relevant for zoological institutions. I found that middle-aged males (3-4 years) produced more motile...
sperm, while older males (8-9 years) had significantly longer sperm. Males of different ages did not differ in sperm concentration or spermic urine volume. Within a zoological setting, this information becomes especially valuable as our results highlight that older males may not be costly to breed and may in fact facilitate successful propagation.

**Age Limitations**

In our study, I choose to categorize males into three age groupings to test the effects of age on sperm quality metrics. Among zoos, age was a limiting factor as there were no frogs available between the age ranges: 5-6 years old and 10-11 years old. While there is a volume of literature on senescence across non-human taxa, research often displays data that compares age ranges spanning a small portion of an organism’s total lifetime. Studies show data collected during an organism’s juvenile years, middle-aged years, or later years (see review, Johnson & Gemmell, 2012). To date, the most comprehensive study was in Barn swallows (*Hirundo rustica*; Møller et al., 2009), which evaluated sperm quality across males aged 1-6 years old. This study found that sperm quality generally declines with male age and evaluated age ranges as a normal distribution across the species relative lifespan (Møller et al., 2009). When studies only focus on a portion of an organism’s lifespan they may predict a reproductive pattern that is not true. One strength of our study is its comprehensive representation of age ranges that span the Mississippi gopher frog’s natural lifespan. However, if I was to re-do this study, I would try to incorporate animals from additional age categories representing the Mississippi gopher frog’s lifespan in captivity (1-11 years old). This would provide a more comprehensive overview of age to benefit captive breeding programs.
**Implications: Potential Downstream Consequences of Age**

Senescence literature suggests an age-dependent effect in male reproductive success is likely to be accompanied by a reduction in fertilization success and offspring longevity (see review, Johnson & Gemmell, 2012). For example, in male fowl (*Gallus gallus domesticus*) an age-dependent decline in sperm velocity and fertilization success was observed (Dean et al., 2010). Considering this, I collected data using a split-clutch *in-vitro* fertilization design to evaluate potential downstream effects of age on fertilization, and hatching success. However, I was restricted by sample size and I was only able to make a qualitative speculation based on the available data. In future breeding seasons, I will continue to collect data using the same split-clutch breeding design to quantitatively evaluate age-dependent effects on fertilization success and offspring fitness. In senescence theory, there has only been a handful of studies that evaluated downstream consequences of age in non-human taxa (Johnson & Gemmell, 2012). The exact mechanism(s) driving an age-dependent decline in fertilization success and offspring longevity are not fully understood. However, oxidative stress is thought to be the most likely cause (Siva-Jothy, 2000). When reactive oxygen species (ROS) accumulate, there is often a positive correlation with DNA fragmentation in the sperm cell and this is linked to a higher level of abnormal sperm development (Aitken et al., 2010). This can have downstream consequences to not only affect fertilization success but may negatively affect offspring viability. For example, Serre & Robaire (1998) showed progeny of older males in the Brown Norway rat (*Rattus norvegicus*) had a significantly higher neonatal mortality. Future studies could choose to evaluate additional sperm quality metrics, such as sperm abnormalities and sperm ultrastructure using a
scanning electron microscope to identify the proportion of spermatozoa that has cellular
damage or abnormalities, which may impact fertilization and offspring longevity.

*Future Directions: Epigenetics*

In recent years, interest has grown in studying epigenetic effects as information is
not limited to coded DNA but can also be transferred through non-genetic inheritance.
Epigenetic effects have been well studies in females, however, paternal effects are far
less understood. Although, there is evidence to suggest that sperm-mediated epigenetics
may play a role in early embryo development and may have consequences to offspring in
later life (Zajitschek et al., 2014). Epigenetic factors include DNA methylation, histone
modifications, and non-coding RNAs, which often drive underlying cellular mechanisms
for ageing in the male germline (Curley et al., 2011). Sperm-mediated epigenetic effects
can be influenced by environment, such as high-level sperm competition. For example,
Zajitschek et al. (2014) showed male zebrafish (*Danio rerio*) exposed to higher levels of
sperm competition produced higher quality sperm and offspring with shorter
development periods. However, offspring longevity was significantly reduced in
comparison to those produced from males in low competition environments (Zajitschek
et al., 2014). Future studies in the Mississippi gopher frog could investigate sperm-
mediated epigenetic effects to identify potential downstream consequences of age on
offspring fitness.

*Chapter 3*

In captive breeding programs, exogenous hormones have been used to overcome
reproductive dysfunction and induce a spermiation response in a variety of anurans
This chapter examined sperm quality following an exogenous luteinizing hormone-releasing hormone analog (LHRHa) injection across different sampling times. The goal of this chapter was to evaluate the spermiation response after an LHRHa treatment to identify an ideal sampling time that produced the highest quality sperm to optimize fertilization success. We injected each male with 0.5μg/g body weight of LHRHa and sampled sperm at 30 minutes, 60 minutes and 120 minutes post-hormone injection. Sperm quality was assessed using four different metrics: progressive motility (%), motility (%), velocity (μm/sec), and concentration (x10^6 cells/ml). We found that sampling time post-LHRHa injection had a significant effect on sperm motility and velocity. Sampling time had a marginal effect on sperm concentration and there was no significant difference in progressive motility.

**Comparison of Exogenous Hormones**

In captive breeding programs, both LHRHa and hCG are often employed to induce gamete production (Kouba et al., 2012). While numerous studies have examined the efficacy of exogenous hormones in anurans, the results of this study are the first to evaluate differences in sperm quality across post-injection sampling time in the Mississippi gopher frog. Our study was limited to using LHRHa to induce a spermiation response in males and would have benefited from testing the efficacy of hCG or a combination of both exogenous hormones simultaneously. Several studies have used hCG or a combination of hormones to collect spermic urine in anurans (Obringer et al., 2000, Rowson et al., 2001; Kouba & Vance, 2009; Mann et al., 2010; Shishova et al., 2011; Uteshev et al., 2013). Kouba & Vance (2009) conducted a study which
characterized sperm production following administration of hCG (500IU) and LHRH (15µg) in the Northern leopard frog (*Rana pipens*). Results indicated that peak sperm production occurred between 30 – 60 minutes following hormone treatment (Kouba & Vance, 2009). Interestingly, numerous studies found that hCG produced a greater spermiation response than in combination with LHRH, including the African clawed frog (*Xenopus laevis*; Easley et al., 1979), the American bullfrog (*Rana catesbeiana*; Easley et al., 1979), the Northern Leopard frog (*Rana pipens*; Waggener & Carroll, 1998a), the Wyoming toad (*Anaxyrus baxteri*; Browne et al., 2006), and the American toad (*Anaxyrus americanus*; Kouba et al., 2012b). Future research could test the efficacy of hCG and LHRH independently, and in combination to observe which treatment elicits a superior response.

*Sperm Quality Limitations*

In Chapter 3, I analyzed four sperm quality metrics that may be linked to fertilization success. However, different sperm quality metrics, such as viability, sperm morphology, and spermic urine volume may also be indicators of fertilization and should be considered in future studies. First, sperm viability – the proportion of live sperm – is a useful metric to analyze when conducting artificial fertilizations (Dziminski et al., 2009; Poole & Grow, 2012). Artificial fertilizations are typically performed by hormonally inducing males and collecting spermic urine samples simultaneously. When spermic urine is collected, sperm is active and motile (Poole & Grow, 2012). The length of time between collection and *in-vitro* fertilization (IVF) will predict how long sperm can be stored before use. If a large proportion of sperm are non-motile, this may affect
fertilization as sperm will not be able to reach a fertile ovum. Secondly, the percentage of abnormal sperm (morphology) is an important indicator of fertilization success (Togna et al., 2017). Sperm abnormalities can arise in both the sperm’s head and flagellum and may influence swimming ability (Poole & Grow, 2012). In this study, I collected samples for sperm morphology analyses, however, I was unable to successfully process samples. Upon examination, sperm were absent of their tails, which were most likely cleaved during processing. Lastly, the volume of spermic urine is important as urine needs to be evenly distributed across eggs during IVF. A minimum of 100µl of spermic urine is recommended to cover approximately 100 eggs. While spermic urine is not the most important predictor of fertilization, if low volumes are collected, then eggs may not be properly fertilized during IVF. Future research could characterize how these additional metrics vary across sampling time to determine any differences which may influence fertilization.

Implications: Fertilization Success

In this study, we characterized four different sperm quality metrics that may improve fertilization success, however, we did not actually test this. Following exogenous hormone treatment, gametes from males and females are collected for IVF (Kouba & Vance, 2009). In captivity, IVF is done using a dry fertilization technique that involves covering eggs in spermic urine, waiting 5 minutes, and then immersing the eggs in water (Poole & Grow, 2012). The simplicity of the IVF protocol makes identifying fertilization success fairly easy, as cleavage of an embryo will typically begin within 4-5 hours (Gosner, 1960; Poole & Grow, 2012). Future studies could conduct artificial
fertilization trials to quantify fertilization success and would help zoos by investigating which sperm quality metric(s) is most valuable to predict fertilization success. A future study could incorporate a competitive fertilization design similar to Dziminski et al. (2009) to evaluate sperm quality per male and create competitive fertilization trials. In creating competitive environments, one could determine paternity through microsatellite analyses and predict which sperm quality metric(s) offered a competitive advantage (Dziminski et al., 2009). This type of study would be valuable for zoos, as IVF techniques often include sperm batching, which is performed when the volume of spermic urine collected from one male is too low for fertilization.

**Conclusions**

In this thesis, my primary research focus was to address a potential cause of reproductive dysfunction in captivity and to evaluate the effect of an assisted reproductive technology to improve breeding protocols for the critically endangered Mississippi gopher frog. I found that male age and sampling time post-hormone injection had a significant effect on sperm quality. Taken together, these results have important implications for captive breeding as zoos may benefit from incorporating these findings into their breeding protocols. Since amphibians have a diversity of reproductive strategies and life histories, it may be that this research is only applicable to closely related species. Thus, this research can be used as a stepping stone to encourage future studies to investigate ways to benefit captive breeding and reintroduction efforts. The Mississippi gopher frog is just one species of many that require assistance to improve the efficacy of their program. This contribution and future research will hopefully lend aid to the
ongoing amphibian extinction crisis and provide strength to the zoos mission to conserve and protect them.
References


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Rowson, A. D., Obringer, A. R., & Roth, T. L. (2001). Non-invasive treatments of luteinizing hormone-releasing hormone for inducing spermiation in American (Bufo americanus) and Gulf Coast (Bufo valliceps) toads. Zoo Biology. 20(2): 63-74


Methods

Hormone Induction of Egg Production & Collection

A total of 7 female Mississippi gopher frogs (*Lithobates sevosus*) housed at the National Amphibian Conservation Center (Detroit Zoo, Royal Oak, MI, USA) were used in this study (see appendix 1.1). Females were given weight specific doses of exogenous hormones to produce eggs (see Poole & Grow, 2012). Each female received two priming doses and one ovulatory dose by intraperitoneal injection. Two priming doses of 3.3IU/g body weight of Human Chorionic Gonadotrophin (hCG) were administered on an arbitrarily selected day (day one) and (day four), ensuring there was seventy-two hours separating each dose. An ovulatory dose of 10IU/g body weight hCG (cat#: C1063; Sigma-Aldrich, St. Louis, Missouri, USA) and 0.5 micrograms/g body weight luteinizing hormone-releasing hormone analog (LHRHa) (cat#: L4513; Sigma-Aldrich, St. Louis, Missouri, USA) was administered 24h following the second primary dose. Each female was placed into a separate holding container filled with approximately 2.54 cm of amphibian safe water following each hormone injection.

Females were checked twice daily for egg production. At each collection attempt, females were grasped with their rear legs pulled up against their body. Gentle pressure was applied to their body for no longer than 30 seconds and an inoculation probe – a plastic rode with a rounded end – was inserted into the cloaca to release any pressure. If females did not release free flowing eggs, they were returned to their enclosure. If eggs were expelled, gentle pressure to their abdomen was applied for a short period of 30
seconds or less, for no longer than five minutes. Eggs were collected into a dry petri dish (1808.48 cm$^3$) until females showed no signs of free-flowing eggs. Once no eggs remained, females were placed into their enclosure and were not reused in the study.

*Hormonal Induction of Spermiation & Collection*

A total of 6 male Mississippi gopher frogs (*Lithobates sevusus*) housed at the National Amphibian Conservation Center (Detroit Zoo, Royal Oak, MI, USA) were used in this study (see appendix 1.1). Each male received an intraperitoneal injection of 0.5µg/g body weight of a LHRHa (cat#: L4513; Sigma-Aldrich, St. Louis, Missouri, USA). Immediately following hormone injection, male Mississippi gopher frogs were placed into holding containers filled with approximately 2.54 cm of amphibian safe water to cover the bottom of the container. This allowed frogs to replenish their bladders between collection times.

Spermic urine samples were collected at one-hour post-injection. Prior to collecting urine, the posterior end of each animal was patted dry using a paper towel to prevent excess water from diluting the sample. Animals were held over a wide petri dish (1808.48 cm$^3$) and a soft piece of catheter tubing (#BB31785-V/5; Scientific Commodities Inc, Lake Havasu City, AZ) was inserted into the cloaca of each male drawing spermic urine into the petri dish. Immediately following urination, the sample was pipetted into a 1.5mL Eppendorf tube (#05-408-129; FisherScientific, Pittsburgh, PA) and placed in a chilling block (#IC22; Torrey Pines Scientific, Carlsbad, CA) set at 4°C.
Experimental Design

Artificial inseminations by in-vitro fertilization were completed at three time points: June 6th, 2018 (n = 5), June 28th, 2018 (n = 1) and February 4th, 2019 (n = 3). Seven female Mississippi gopher frogs produced 1386 eggs, that were collected by clutch in separate petri dishes identified by female ID. Each clutch (n = 9) was further separated into two petri dishes and artificially crossed with of two different males (i.e. relatively young and relatively old). This split-clutch in-vitro fertilization design (see results; appendix 1.2) was completed to ensure each maternal half-sib-ship created a full sib-ship sired by a relatively old male and a relatively young male.

In-vitro Fertilization

Artificial fertilizations were performed using a dry in-vitro fertilization technique (Poole & Grow, 2012). At peak sperm concentration, approximately at 1-hour post-hormone injection (see Watt et al., 2019: in review), 50µl - 200µl of spermic urine from a randomly selected male was pipetted evenly onto a female’s eggs. The volume of spermic urine used for fertilization was dependent on the volume of spermic urine produced by each male. Eggs were left standing without water for a five-minute period measured by a stopwatch timer. Once five minutes had elapsed, eggs were gently flooded with amphibian safe water (~18°C) until all eggs were completely submerged.

Fertilization and Hatching Success

Fertilization rates were determined within 4-5 hours following in-vitro fertilization. Amphibian eggs consist of two poles: a dark colored animal pole and a light-colored vegetal pole (Altig & McDiarmid, 2007). When fertilized, the dark animal pole will rotate upward, and fertilization rates can be visually counted by the percentage of
eggs that are fully or partially displaying the animal pole. However, Mississippi gopher frog eggs do not possess a strong dichromic separation of the poles, and fertilization rates were confirmed using a handheld digital microscope pro (Celestron). This instrument allowed for a high-resolution image of the ova pigmentation to become visible and each clutch was photographed. Fertilization rates were calculated as the number of eggs per clutch with the dark animal pole rotated upward, divided by the number of eggs unrotated, multiplied by 100. Hatching rate (stage 20; Gosner, 1960) occurred approximately five days post-fertilization at (~18°C) and was recorded as the number of individuals outside their egg capsule. All hatchlings were visually counted using a direct counting method by one observer.

**Analysis & Results**

In appendix 1.2, we present raw data for each split-clutch replicate and the corresponding fertilization and hatching success between young and old male crosses. The mean (± standard error) fertilization and hatching success between young and old male crosses was calculated (see appendix 1.3). Mean fertilization success for young males was 32.54 ± 8.16 and in old males was 28.91 ± 6.72. Hatching success for young males was 7.91 ± 5.35, compared to old males 4.27 ± 3.88. To investigate this relationship further, a spearman’s rho correlation was calculated for fertilization success. We investigated the relationship between fertilization success and young males and the relationship between fertilization success and old males. We also investigated the relationship between hatching success and young males and the relationship between fertilization success and old males. There was no association found between fertilization success and young males ($r_s = 0.54, P = 0.084$). However, there was an association found
between fertilization success and old males ($r_s = 0.78$, $P = 0.004$). There was no association found between hatching success and young males ($r_s = 0.35$, $P = 0.28$) or old males ($r_s = 0.13$, $P = 0.70$).

References


Appendix 1.1  Summary table of Mississippi gopher frogs age (years), snout-vent length (mm) and body weight (g). Animal ID corresponds to individuals used in split-clutch study (table 1.2).

<table>
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Appendix 1.2 Split-clutch replicates between young and old male crosses. Information is presented by split-clutch ID and the corresponding female and male pairs and their relative ages. Ages are presented in zoo coding (years.months). Percent fertilized, and percent hatching refer to the number of eggs fertilized or hatched out of the total number of eggs counted per split-clutch.

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<th>Fertilized (%)</th>
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Appendix 1.3 Mean (± standard error) fertilization success between relatively young and old male sire crosses.
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